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THE ROLE OF CATHEPSIN K IN LUNG DEVELOPMENT AND NEWBORN CHRONIC LUNG INJURY

Jonni Knaapi

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University of Turku

Faculty of Medicine
Department of Pediatrics
Institute of Clinical Medicine
Research Centre of Applied and Preventive Cardiovascular Medicine (CAPC)
Doctoral Programme of Clinical Investigation – CLIDP

Supervised by

Docent Heikki Lukkarinen, M.D.
Department of Pediatrics and Research Centre of Applied and Preventive Cardiovascular Medicine.
University of Turku
Turku, Finland

Professor Pekka Kääpä, M.D.
Department of Pediatrics and Research Centre of Applied and Preventive Cardiovascular Medicine.
University of Turku
Turku, Finlandd

Reviewed by

Docent Outi Tammela, M.D.
Department of Pediatrics
University of Tampere
Tampere, Finland

Docent Olli Pitkänen M.D.
Department of pediatrics
University of Helsinki
Helsinki, Finland

Opponent

Professor Mikko Hallman, M.D.
Department of Pediatrics
University of Oulu
Oulu, Finland

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ABSTRACT

Jonni Knaapi: The role of cathepsin K in lung development and newborn chronic lung injury.

Department of Pediatrics, Research Centre of Applied and Preventive Cardiovascular Medicine (CAPC), University of Turku, Turku, Finland; Annales Universitatis Turkuensis, Medica-Odontologica, 2014.

Chronic lung diseases, specifically bronchopulmonary dysplasia (BPD), are still causing mortality and morbidity amongst newborn infants. High protease activity has been suggested to have a deleterious role in oxygen-induced lung injuries. Cathepsin K (CatK) is a potent protease found in fetal lungs, degrading collagen and elastin. We hypothesized that CatK may be an important modulator of chronic lung injury in newborn infants and neonatal mice.

First we measured CatK protein levels in repeated tracheal aspirate fluid samples from 13 intubated preterm infants during the first two weeks of life. The amount of CatK at 9-13 days was low in infants developing chronic lung disease. Consequently, we studied CatK mRNA expression in oxygen-exposed wild-type (WT) rats at postnatal day (PN) 14 and found decreased pulmonary mRNA expression of CatK in whole lung samples. Thereafter we demonstrated that CatK deficiency modifies lung development by accelerating the thinning of alveolar walls in newborn mice. In hyperoxia-exposed newborn mice CatK deficiency resulted in increased number of pulmonary foam cells, macrophages and amount of reduced glutathione in lung homogenates indicating intensified pulmonary oxidative stress and worse pulmonary outcome due to CatK deficiency. Conversely, transgenic overexpression of CatK caused slight enlargement of distal airspaces with increased alveolar chord length in room air in neonatal mice. While hyperoxic exposure inhibited alveolarization and resulted in enlarged airspaces in wild-type mice, these changes were significantly milder in CatK overexpressing mice at PN7. Finally, we showed that the expression of macrophage scavenger receptor 2 (MSR2) mRNA was down-regulated in oxygen-exposed CatK-deficient mice analyzed by microarray analysis.

Our results demonstrate that CatK seems to participate in normal lung development and its expression is altered during pulmonary injury. In the presence of pulmonary risk factors, like high oxygen exposure, low amount of CatK may contribute to aggravated lung injury while sustained or slightly elevated amount of CatK may even protect the newborn lungs from excessive injury. Besides collagen degrading and antifibrotic function of CatK in the lungs, it is obvious that CatK may affect macrophage activity and modify oxidative stress response. In conclusion, pulmonary proteases, specifically CatK, have distinct roles in lung homeostasis and injury development, and although suggested, broad range inhibition of proteases may not be beneficial in newborn lung injury.

Key words: apoptosis, bronchopulmonary dysplasia, inflammation, lung development, cathepsin K

TIIVISTELMÄ

Jonni Knaapi: Katepsiini K, keuhkojen kehitys ja vastasyntyneen krooninen keuhkovaurio

Lastentautioppi, Sydäntutkimuskeskus, Turun yliopisto, Turku; Annales Universitatis Turkuensis, Medica-Odontologica, 2014.

Vastasyntyneen krooninen keuhkovaurio (BPD) on edelleen merkittävä hoidollinen ongelma ennenaikaisesti syntyneillä lapsilla. Katepsiini K on voimakas keuhkoista löydetty proteaasi, joka hajottaa kollageenia ja elastiinia. Keuhkovauriomalleissa on viime aikoina todettu proteaasiaktiviteetin muutoksia, jotka saattavat olla haitallisia keuhkojen kehittymiselle, mutta proteaasien rooli keuhkojen kehittymisessä on vielä epäselvä. Tutkimuksen tarkoituksena oli tutkia katepsiini K:n merkitystä/osuutta keuhkojen kehitykseen ja keuhkoissa tapahtuviin rakenteellisiin muutoksiin korkean happipitoisuuden aiheuttamassa keuhkovauriomallissa hiirillä.

Mittasimme katepsiini K:n pitoisuuksia toistetusti otetuista suurten ilmateiden imulimanäytteistä 13 vuorokauden ajalta kolmeltatoista hengityskonehoidetulta ennenaikaiselta vastasyntyneeltä lapselta. Totesimme katepsiini K:n erityksen alentuneen kroonisen keuhkovaurion kehittäneillä 9-13 vuorokauden kohdalla. Lisäksi totesimme happialtistettujen vastasyntyneiden rottien katepsiini K -lähetti-RNA:n tuotannon laskeneen ja keuhkojen sidekudoksen lisääntyneen. Toiseksi tutkimme katepsiini K -puutteisilta poistogeenisiltä hiiriltä keuhkojen kehitystä ja muutoksia happivaurioon liittyen. Totesimme katepsiini K:n tuotannon keskittyvän keuhkoputkien ja keuhkorakkuloiden seinämiin sekä makrofageihin. Huoneilmassa Katepsiini K:n suhteen poistogeenisilla hiirillä seitsemän vuorokauden kohdalla keuhkorakkuloiden seinämät olivat normaalia ohuemmat. Lisähapessa poistogeenisillä hiirillä pelkistetyn glutationin määrä oli lisääntynyt oxidatiivisen stressin merkkinä. Makrofagien määrä oli näillä lisääntynyt ja solut olivat rasvatäyteisiä. Kolmannessa osatyössä tutkimme katepsiini K:n ylituotannon vaikutuksia ja totesimme 14 vuorokauden kohdalla periferisesti keuhkorakkuloiden koon kasvaneen huoneilmassa hoidetuilla hiirillä. Hapessa keuhkorakkulat olivat laajentuneet 7 vuorokauden kohdalla villityyppihiirillä ja löydökset pahenivat 14 vuorokauden kohdalla. Katepsiini K:n ylituotanto suojeli keuhkojen rakennetta 7 vuorokauden kohdalla mutta keuhkorakkuloiden laajeneminen oli ilmeistä 14 vuorokauden kohdalla. Neljännessä osatyössä totesimme myös hapessa hoidetuilla poistogeenisilla hiirillä macrophage scavenger receptor 2:n (MSR2) –lähetti-RNA:n tuotannon vähentyneen keuhkoissa.

Katepsiini K vaikuttaa keuhkojen kehitykseen syntymän yhteydessä ja saattaa olla merkittävä tekijä kroonisen keuhkovaurion synnyssä muiden riskitekijöiden, kuten happivaurion yhteydessä. Katepsiini K:n puutos vaikuttaisi johtavan pahenevaan keuhkovaurioon kun taas lisääntynyt määrä saattaa jopa suojella vauriolta. Katepsiini K:lla on mahdollisesti antifibroottisten ominaisuuksien lisäksi vaikutusta makrofagien aktiivisuuteen ja happiradikaalien käsittelykykyyn. Proteaasit, kuten katepsiini K, vaikuttavat moninaisesti keuhkojen kehitykseen ja lisää tutkimusta tarvitaan näiden erityisominaisuuksien selvittämiseksi.

Avainsanat: Katepsiini K, krooninen keuhkovaurio (BPD), apoptoosi, inflammaatio, keuhkojen kehitys

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred in the text by Roman numerals I-IV.

I. Knaapi J, Lukkarinen H, Kiviranta R, Steiner A, Lassus P, Andersson S, Kääpä P. Cathepsin K expression is diminished in infants with bronchopulmonary dysplasia. 2006; Acta Pediatr 95(10):1298-300.

II. Knaapi J, Lukkarinen H, Kiviranta R, Vuorio E, Kääpä P. Cathepsin K aggravates lung injury in hyperoxia-exposed newborn mice. 2011; Exp Lung Res 37(7):408-18.

III. Knaapi J, Kiviranta R, Laine J, Kääpä P, Lukkarinen H. Cathepsin K overexpression modifies lung development in hyperoxia-exposed newborn mice. Pediatr Pulmonol 2014; Feb 20. doi: 10.1002/ppul.23011.

IV. Knaapi J, Kiviranta R, Soukka H, Kääpä P, Lukkarinen H. Bioinformatic analysis of hyperoxia-induced gene expression in neonatal mice lungs – the role of cathepsin K deficiency. Manuscript.

ABBREVIATIONS

ANGII	Angiotensin II
BPD	Bronchopulmonary dysplasia
CatK	Cathepsin K
GSH	Reduced glutathione
KO	Knock-out
MPO	Myeloperoxidase
MSR2	Macrophage scavenger receptor 2
PN	Postnatal day
RDS	Respiratory distress syndrome
SAM	Significance analysis for microarrays
TAF	Tracheal aspirate fluid
(TBA- reactive material)	Lipid peroxides
TG	Transgenic overexpressing
WT	Wild type

1 INTRODUCTION

Bronchopulmonary dysplasia (BPD) is still causing considerable morbidity and mortality amongst premature infants (Jobe and Bancalari 2001), and may result in reduced lung capacity even in late adolescence (Bose, Dammann et al. 2008; Hadchouel, Franco-Montoya et al. 2014). Inflammation, impaired alveolar and vascular development and variable fibroproliferation are the main pathological findings in BPD (Coalson 2003). Ventilatory treatment and oxygen supplementation to premature lungs and perinatal infections are known predisposing factors to BPD (Coalson 2003; Bose, Dammann et al. 2008). In fact, hyperoxia has been shown to induce pulmonary inflammation, aggravate fibrosis, and disrupt alveolar development thus creating similar pathological changes to those seen in BPD (Bose, Dammann et al. 2008; Mao, Gundavarapu et al. 2008). Still, mechanisms behind the disrupted vascular and alveolar development in affected newborn lung are unclear.

Increased protease activity is suggested to result in inflammatory and fibroprolifrative changes in newborn lung injury (Ryu, Vicencio et al. 2005). Pulmonary proteases are further proposed to be associated with impaired alveolarization in hyperoxia-induced lung injury (Hirakawa, Pierce et al. 2007; Chetty, Cao et al. 2008). In fact, increased levels of pulmonary cathepsins and metalloproteinases have been measured in the lungs of newborn infants and baboons with developing lung injury (Cederqvist, Sorsa et al. 2001; Altiok, Yasumatsu et al. 2006; Bose, Dammann et al. 2008). However, it seems clear that each protease has a unique role in the development of newborn lung injury and thus the function of each protease should be evaluated.

Cathepsin K (CatK) is a cysteine protease and one of the most potent collagenases and elastases remodeling extracellular matrix by degrading type I, II and IV collagen and elastin (Chapman, Riese et al. 1997; Buhling, Waldburg et al. 2000). CatK was first found in osteoclasts but has later also been found in the normal lungs of infants and newborn mice (Rantakokko, Aro et al. 1996; Buhling, Waldburg et al. 2000; Buhling, Waldburg et al. 2002). In the adult and fetal lungs, CatK is expressed in the bronchial and alveolar epithelial cells (Buhling, Waldburg et al. 2000; Buhling, Waldburg et al.

2002) and is suggested to be related to perinatal lung development and matrix remodelling (Chapman, Riese et al. 1997; Haeckel, Krueger et al. 1999; Buhling, Waldburg et al. 2000; Buhling, Waldburg et al. 2002; Zhang, Leung et al. 2011). CatK is further found to be upregulated at the site of inflammation, possibly thus contributing to inflammatory and fibroproliferative disorders of the adult and newborn lungs (Chapman, Riese et al. 1997; Buhling, Rocken et al. 2004; Bose, Dammann et al. 2008). The role of CatK in the normal developing and affected newborn lung injury is however still unclear.

The main objective of this thesis was to investigate, using newborn wild-type, CatK-deficient and CatK overexpressing transgenic mice and tracheal aspirate fluids of newborn infants developing BPD, if CatK contributes to normal lung development or hyperoxia-induced lung injury.

2 REWIEV OF THE LITERATURE

2.1 Lung development

2.1.1 Prenatal alveologenesis

Normal prenatal lung growth and development in human has been separated in four characteristic periods (Potter and Loosli 1951). Organogenesis mainly occurs in embryonic period from 0 to 7 gestational weeks. Lungs appear fourth gestational week as a ventral bud of esophagus. At sixth week lobar and segmental branches are formed (Burri 1984). Pseudoglandular phase covers 6 to 17 weeks from gestation. All conducting airways develop within this period and acinar outlines are formed (Kitaoka, Burri et al. 1996). Cellular differentiation and growth begins from the proximal to distal end (Merkus, ten Have-Opbroek et al. 1996; Smith, McKay et al. 2010). Airway tubules are lined with high columnar epithelium and at 12 gestational week trachea and segmental bronchi entail cartilage and smooth muscle cells, the fluid secreting glands and cells develop and distally sparse differentiation to cuboidal epithelium progress (Burri 1984; Merkus, ten Have-Opbroek et al. 1996). Distal cuboidal cells contain glycogen which appears in the site of cell differentiation and is an essential component for surfactant. Cuboidal cells are immature type II epithelial cells which will eventually secrete surfactant in saccular phase to reduce surface tension and prevent alveoli from collapse (Burri 1984). In weeks 17 to 26 lungs, are under the canalicular phase of development. Acinus grow via peripheral branching of the primitive airway (Burri 1984). Widening of distal airspace converge it to cuboidal cells and flattens, further forming blood-air barrier (Burri 1984). Formation of blood air barrier begins distally when capillaries modulate overlying epithelial cells to type I pneumocytes (Mercurio and Rhodin 1976). Distally type II epithelial cells gradually release surfactant until the end of this period, but steady surfactant formation becomes stable at around 26 gestational week (Burri 1984). In the saccular period from 24 gestational week to 40 gestational week, airways evolve to thin walled terminal saccules (Burri 1984). During the saccular period there is natural increase in system cortisol concentrations which is prudent for normal

lung development and particularly it is critical to preparing lungs for normal gas exchange after birth (Strang 1991; Jobe and Ikegami 2000). Lung development in humans and rodents share similar developmental phases. The duration of each period is related to gestational time which is18-20 days in mice and 40 weeks in humans (Amy, Bowes et al. 1977; Burri 1984; Burri 2006).

2.1.2 Prenatal pulmonary angiogenesis

Pulmonary angiogenesis proceeds concurrently with alveolar development (Smith, McKay et al. 2010). Insufficient pulmonary angiogenesis has been shown to lead in growth arrest resulting in poorly developed lungs with enlarged alveoli (Thebaud 2007). During fetal development there are variations in vessel length and diameter but not in density (Burri 2006). There are two definitions for vessel formation 1) in vasculogenesis new vessels differentiate from endothelial cells (Hislop and Reid 1972) and 2) angiogenesis generates new vessels branching from capillaries (Risau and Flamme 1995). During the embryonic period at sixth gestational week pulmonary arteries are budding from the sixth aortic arch and pulmonary veins formed from heart (Burri 1984). Pre-acinar vascular bed, comprising veins and arterial tree, is completed until the end of pseudoglandular phase and bears resemblance with architecture of adult lungs (Hislop and Reid 1972; Burri 1984). In the canalicular period capillaries are sprouting between flattened epithelial cells making gas exchange possible at birth (Campiche, Gautier et al. 1963). Generally, in this period vascular development consists of proliferation and organization of vessels to network around airspaces (deMello and Reid 2000). Characteristically in saccular period vessels are growing in diameter and length and new vessels are formed along with airways expansion (Burri 1984; deMello and Reid 2000). Lung vascular growth due to developmental stages are very alike in mammals but the duration of the stages varies according to the gestational length (Pinkerton and Joad 2000).

2.1.3 Postnatal lung development

Pulmonary gas exchange is managed by the placenta before birth but perinatally this process is transferred to the lungs where the abundant vascular bed is excellent

environment for gas exchange (Sansoucie and Cavaliere 1997). After birth, the development of the human lungs proceeds to alveolar period (from gestational age 36 weeks to 2 years post term) (Burri 1984). Despite being functional the remodeling of the parenchyma and capillaries within the lungs continue and 85 % of alveolarization occurs after birth (Smith, McKay et al. 2010). Alveolarization is divided to three phases (Smith, McKay et al. 2010). First appears the secondary septa with increased surfactant expression (Burri 1984). Then the double lumen capillaries are modified to single lumen system and the alveolar septae are thinned and elongated (Burri 1984). Finally (after 2 years), all the lung components grow until growth of the long bones cease (Smith, McKay et al. 2010). Newborn mouse lungs are in the saccular phase at the time of birth and alveolarization occurs between days 5 and 28 postnatally while pulmonary development in human infants proceeds to alveolar period at 36 gestational week (Amy, Bowes et al. 1977; Burri 2006; Bhandari 2014).

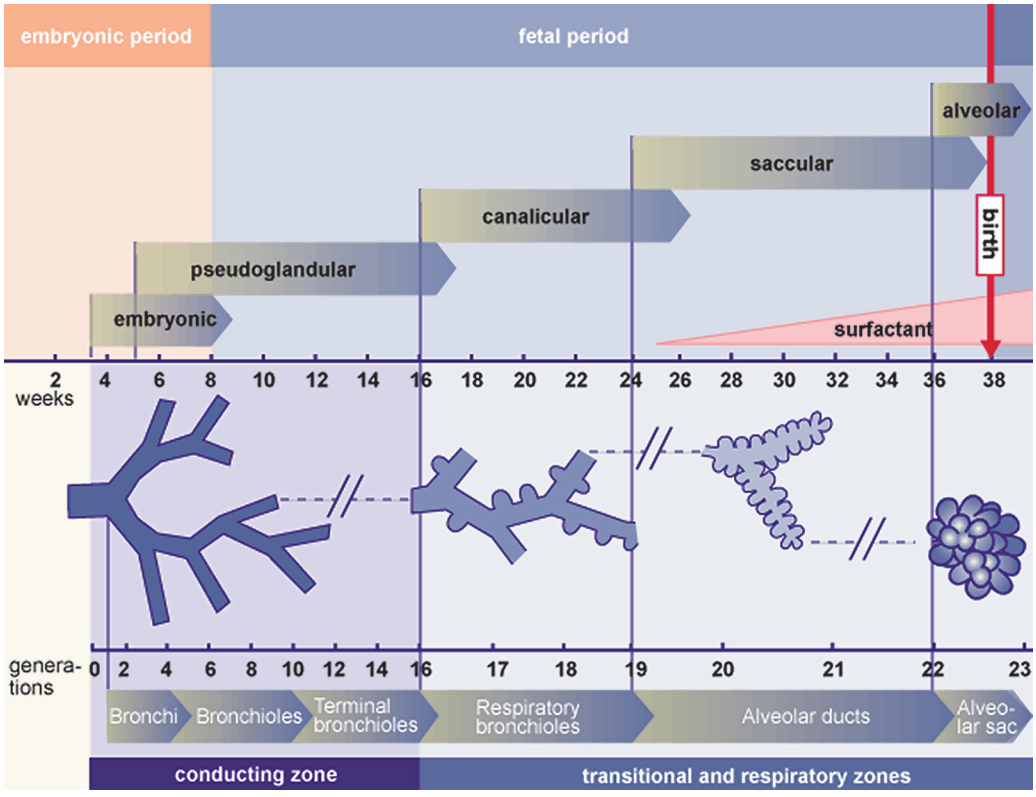

Figure 1: Prenatal human lung development (Burri 1984).

2.2 Bronchopulmonary dysplasia (BPD)

BPD is a chronic lung disease causing the most short- and long-term morbidity and mortality in newborn infants (Cotten, Oh et al. 2005; Bose, Dammann et al. 2008). Incidence of BPD has been shown to vary between 10-50 % globally, depending on the studies, within under 1250g birth weight premature infants (Eichenwald and Stark 2008; Jensen and Schmidt 2014). Incidence rates of BPD have remained stable or even increased in the last two or three decades due to increased survival of the more premature infants (Jensen and Schmidt 2014).

BPD is nowadays commonly divided into two forms: old and new BPD. Old BPD was mainly affecting infants suffering from respiratory distress syndrome (RDS) and poorly developed lungs with surfactant deficiency after ventilator treatment (Northway, Rosan et al. 1967; Mosca, Colnaghi et al. 2011). It was characterized by smooth muscle hyperplasia, fibrosis and specific x-ray findings (Northway 2001; Mosca, Colnaghi et al. 2011). After surfactant was discovered and used in the treatment of distressed infants, incidence of old BPD has diminished (Mosca, Colnaghi et al. 2011). Whereas advancement in neonatal care during this period resulted in increased survival of premature infants, their lung development was still interrupted resulting in a new form of BPD. This new post-surfactant BPD is characterized by impaired alveolar and vascular development, variable fibroproliferation and inflammation in injured lungs (Coalson 2003).

BPD is clinically defined as a need of mechanical ventilation, continuous positive airway pressure (CPAP) or supplemental oxygen need at 35-37 correlated gestational week (Jobe and Bancalari 2001; Walsh, Yao et al. 2004). In clinical practice the severity based consensus defines BPD as a chronic lung injury encountered in infants born under 32 gestational weeks. Assessment is made at 36 weeks of postmenstrual age or when the patient is discharged home. Oxygen therapy over 21 % for at least 28 days is required for the diagnosis. In mild BPD the infant is breathing room air at assessment time. Moderate BPD is defined as oxygen therapy under 30 % and severe BPD as oxygen therapy at least 30 % and/or positive pressure (Ehrenkranz, Walsh et al. 2005).

The pathogenesis of BPD is complex, but hyperoxia, prolonged ventilatory treatment, prematurity and perinatal infections are all still identified as significant risk factors in both forms of BPD (Ambalavanan and Carlo 2004; Chess, D'Angio et al. 2006; Bose, Dammann et al. 2008) (Figure 2). Perinatal dysregulation of cytokine responses is additionally suggested to predispose to BPD and it could serve as a target for new therapies preventing BPD in the future (Paananen, Husa et al. 2009). Regardless of the advancements in the means of respiratory support of the premature newborn infants, oxygen challenge is still a major pathogenetic factor exacerbating development of BPD. Genetic alterations are also connected to preterm birth and the development of BPD (Parton, Strassberg et al. 2006; Prows, Hafertepen et al. 2007) but understanding of genetic interactions in normal and abnormal development remains to be clarified (Hallman 2012). BPD remains one of the most lethal diseases in newborn infants and further research is needed, while prevention of premature births would eventually be the best way to avoid chronic lung diseases (Hallman 2012).

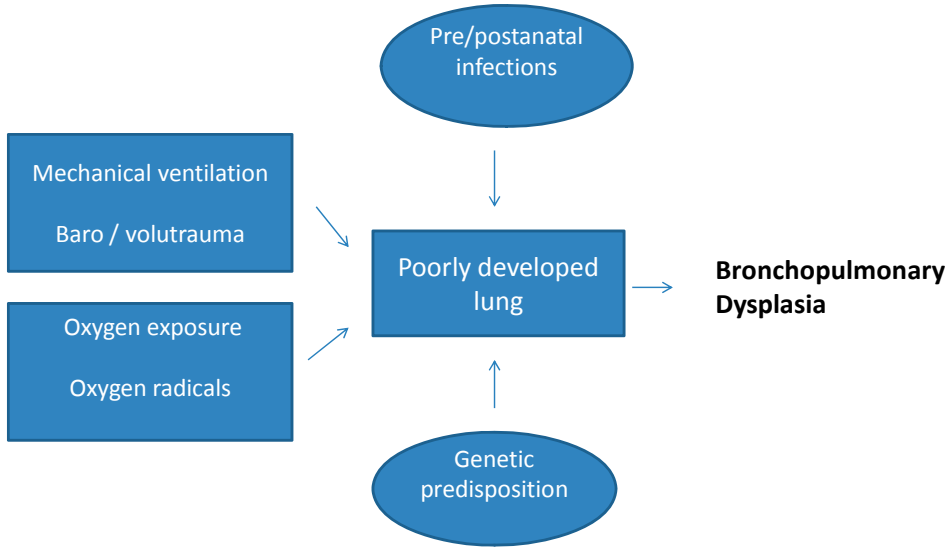

Figure 2: Development of human bronchopulmonary dysplasia. Modified from the picture produced by Thompson A et al. (Thompson and Bhandari 2008).

2.3 Experimental models of chronic lung injury

Different experimental models of chronic lung injury have been used to emulate development of BPD including antenatal exposure to endotoxins with or without oxygen (Tang, Seedorf et al. 2010), perinatal pulmonary IL-1β production (Bry, Whitsett et al. 2007) and oxygen exposure (Bonikos, Bensch et al. 1975). Suitable animal models include rodent, rabbit, lamb and baboon (Buczynski, Maduekwe et al. 2013). Baboons are delivered after 75 % completion of gestation and after birth treated with ventilator treatment with 100 % oxygen for 7 days and after that 85 % oxygen for 14 days (Coalson, Winter et al. 1995). While the pathophysiological mechanisms between these injury models may be different, the morphological outcome is fairly similar – disruption of the lung parenchymal structure and poor alveolarization. Common injury model in mice mimicking newborn lung injury is the hyperoxia model (Bhandari 2014). Hyperoxic challenge of the newborn lungs are shown to induce pulmonary cellular death, fibroproliferative changes and inflammation, and to disrupt alveolar development resulting in enlarged emphysematic airspaces creating pathological alterations that are very similar to those in BPD (Pappas, Obara et al. 1983; Bose, Dammann et al. 2008; Mao, Gundavarapu et al. 2008). The presence of alveolar atelectasis, pulmonary edema and pleural effusion are common findings in oxygen exposed animals (Dickerson 1964; Bonikos, Bensch et al. 1975; Pappas, Obara et al. 1983; Warner, Stuart et al. 1998). There is also evidence that hyperoxic exposure results in pulmonary artery remodeling and pulmonary hypertension (Jones, Zapol et al. 1984). However, oxygen tolerance has been shown to be strain specific (Whitehead, Burch et al. 2006). The control mechanisms of alveolar development are still incompletely understood and the molecular mechanisms linking hyperoxic exposure to disrupted alveolarization remain unclear.

2.4 Pathogenesis of hyperoxia-indused lung injury

2.4.1 Pulmonary Apoptosis

Apoptosis is defined as programmed cell death that may occur in multicellular organisms. Normal postnatal lung development is characterized by gradual thinning of

the distal airspaces (Galambos and Demello 2008), supposedly due to enhanced pulmonary apoptosis on the first postnatal days (Kresch, Christian et al. 1998; Galambos and Demello 2008). Prolonged hyperoxia (O_2 >90%) may induce apoptotic and non-apoptotic cell death in neonatal mice and increased apoptosis may disrupt alveolarization (Pagano and Barazzone-Argiroffo 2003; De Paepe, Gundavarapu et al. 2008; Mao, Gundavarapu et al. 2008). However, these reactions also depend on the genetic background of the mice (Whitehead, Burch et al. 2006). Prolonged hyperoxia has been shown to induce both apoptotic and non-apoptotic cell death and decrease pulmonary cell proliferation in the lungs of neonatal mice during the first week of life which is a critical period in alveolar septation (Galambos and Demello 2008; Mao, Gundavarapu et al. 2008). Pulmonary apoptosis has been shown to increase at postnatal day 3 in hyperoxia-exposed murine lungs (McGrath-Morrow and Stahl 2001), but it ceases by the postnatal day 7 (Husari, Dbaibo et al. 2006). However, it has been suggested that anti-apoptotic strategies may not attenuate alveolar damage in oxygen induced lung injury (Barazzone, Horowitz et al. 1998). Destruction of epithelial cells by programmed cell death is poorly known but for example apoptosis has been suggested to be mediated by angiotensin II (ANG II) receptor subtype 1 in surfactant depleted rats (Lukkarinen, Laine et al. 2004; Lukkarinen, Laine et al. 2005).

2.4.2 Oxidative stress of the lungs

Oxidative stress has shown to be associated with inflammatory response (Pitkanen, Hallman et al. 1991; Fellman and Raivio 1997). Prolonged hyperoxic exposure may result in pulmonary injury via uncontrolled excessive immunologic response which has been shown to be aggravated by ventilatory treatment or perinatal infections (Bose, Dammann et al. 2008). Reactive O_2 species (ROS) are widely produced under hyperoxic conditions (Pitkanen, Hallman et al. 1991; Pagano and Barazzone-Argiroffo 2003). The ROS elevation in hyperoxia has been shown to result in cell and tissue damage via disrupted oxidant antioxidant homeostasis (Rahman, Biswas et al. 2006; Clanton 2007; Ciencewicki, Trivedi et al. 2008). Fraction of inspired O_2, atmospheric pressure and time of exposure determine the cumulative O_2 dose resulting in toxicity. Extended exposure (>24 hours) to over 60 % oxygen consumption in normal

barometric conditions is toxic for the lungs. Oxygen exposure leads to pulmonary edema, airway congestion and bronchoalveolar damage dependent atelectasis. Pulmonary edema results in shortness of breathing (Mason R 2005). In line with previous findings, reactive oxygen species predispose the lungs to alveolar flooding, hemorrage and collagen, elastin and hyaline membrane deposits (Pagano and Barazzone-Argiroffo 2003; Kang, Lee et al. 2005; Yee, Vitiello et al. 2006). Prolonged oxidative stress due to hyperoxia may increase the quantity of reduced GSH to prevent oxidative damage in the lungs (Chessex, Lavoie et al. 1999; Rahman and MacNee 2000; Rahman, Biswas et al. 2005). GSH is, on the other hand, supposed to conserve proteolytic activity of CatK (Lecaille, Bromme et al. 2008).

2.4.3 Lung Inflammatory cells

Accumulation of inflammatory cells in poorly developed lungs is a prominent feature in the development of newborn lung injury (Clement, Chadelat et al. 1988; Warner, Stuart et al. 1998; Speer 1999; Vozzelli, Mason et al. 2004; Speer 2006). These cells are a major source for potent proteases, cytokines and toxic oxygen radicals, which may disrupt normal postnatal alveolarization and pulmonary vascular development in the affected lungs (Chapman, Riese et al. 1997; Speer 1999; Jankov, Johnstone et al. 2003). An imbalance in proinflammatory and anti-inflammatory mediators leads to activation of cellular death pathways in the lungs, which is followed by repair process (Bhandari and Elias 2006; Bhandari and Bhandari 2009). The role of invading macrophages is still unclear. It has been shown that inhibition of macrophage influx improve pulmonary outcome in hyperoxia-induced lung injury in newborn rats (Vozzelli, Mason et al. 2004) as well as ventilator-induced lung injury in adult rats (Eyal, Hamm et al. 2007). However, macrophages are essential for resolution of inflammatory lung injury (Cox, Crossley et al. 1995; Hussain, Wu et al. 1998), and macrophage deficiency may even worsen pulmonary outcome after inflammation (Miyake, Kaise et al. 2007). On the other hand, prolonged hyperoxic exposure result in pulmonary injury via uncontrolled excessive immunologic response which has been shown to be aggravated by ventilatory treatment or perinatal infections (Bose, Dammann et al. 2008). There is also evidence that macrophage activation in newborn

mice results in impaired alveolar development and decreased survival (Blackwell, Hipps et al. 2011).

2.4.4 Implications from the studies of atherosclerosis

Foamy macrophages are not constant characteristic finding in the newborn lung injury, but these cells are present in lung granulomas of adult lungs (Ordway, Henao-Tamayo et al. 2005) and especially in the inflamed perivascular areas of atherosclerotic plaques (Gerrity and Naito 1980; Charo and Taubman 2004). Macrophage scavenger receptor II (MSR II) and scavenger receptor B (CD36) are macrophage scavenger family members, which both have a major role in foam-cell formation by oxidized low-density lipoprotein (LDL) influx (Tabas, Williams et al. 2007). Knock-out of either results in reduced LDL intake (Tabas, Williams et al. 2007). MSR II-deficiency results in 32% decrease in atherosclerotic plagues in atherosclerotic mice model but the knock-out of both MSR II and CD36 did not have further benefits (Kuchibhotla, Vanegas et al. 2008). Foamy macrophages are generally suggested to be unwanted injurious cells in the tissue, generating high rates of reactive oxygen species (Rajagopalan, Meng et al. 1996).

2.4.5 Pulmonary Fibrosis

Slight pulmonary fibrosis affects newborn infants with BPD (Jobe and Bancalari 2001). Collagen accumulation have been shown to commonly increase with time in oxygen exposed newborn rats after 21 days but not before (Chen, Wang et al. 2007). Persistent inflammation results in aggravated pulmonary fibrosis mediated by fibroblasts gathering to repair the damaged lungs. Fibrosis may also be induced by destroying type II epithelial cells suggesting that the disruption of type II epithelial cells may suppresses anti-fibrotic capacity (Sisson, Mendez et al.). Fibrosis and scarring lead to deteriorated gas exchange and short- and longterm impaired lung function lasting into late adolescence (Bose, Dammann et al. 2008).

2.5 Proteases in chronic lung injury and bronchopulmonary dysplasia

Increased activity of proteases is connected with tissue inflammatory and fibroproliferative damage in newborn lung injury (Ryu, Vicencio et al. 2005). Similarly, in newborn hyperoxic lung injury models pulmonary proteases are suggested to impair alveolarization (Chetty, Cao et al. 2008). Thus, imbalance between proteases and antiproteases is associated with the pathogenesis of BPD (Speer 2006). In agreement, elevated pulmonary levels of matrix metalloproteases and cathepsins have been found in newborn infants and baboons with a developing chronic lung injury (Cederqvist, Sorsa et al. 2001; Ryu, Vicencio et al. 2005; Altiok, Yasumatsu et al. 2006; Bose, Dammann et al. 2008). Although high protease activity is suggested to be injurious for inflamed newborn lungs (Cederqvist, Sorsa et al. 2001; Hirakawa, Pierce et al. 2007; Lukkarinen, Hogmalm et al. 2009; Bry, Hogmalm et al. 2010), the unique role of each protease in the development of newborn lung injury still remains controversial (Table 1).

Table 1: Proteases in bronchopulmonary dysplasia (BPD) and experimental models of chronic lung injury. Arrows show up- or downregulations of proteases in BPD and chronic lung injury. Tracheal aspirate fluid (TAF), Matrix metalloproteinase (MMP), Tissue inhibitor of metalloproteinase (TIMP), cathepsin (Cat).

Proteases in BPD and experimental models of chronic lung injury			
BPD			
MMP 8	↑	TAF	(Cederqvist, Sorsa et al. 2001)
MMP 9	↑	Cord blood,TAF	(Schock, Sweet et al. 2001; Fukunaga, Ichiyama et al. 2009)
MMP 9/TIMP 1	↑	Cord blood, TAF	(Ekekezie, Thibeault et al. 2004; Fukunaga, Ichiyama et al. 2009)
TIMP 2	↓	TAF	(Cederqvist, Sorsa et al. 2001)
CatK	↓	TAF	(Knaapi, Lukkarinen et al. 2006)
Trypsin-2	↑	TAF	(Cederqvist, Haglund et al. 2003)
Chronic lung injury			
MMP2	↑	Rat, Mouse	(Pardo and Selman 1996; Zhang, Zhu et al. 2008)
MMP8	↓	Rat	(Fu and Xue 2007)
MMP9	↑	Baboon, Mouse	(Tambunting, Beharry et al. 2005; Zhang, Zhu et al. 2008; Lukkarinen, Hogmalm et al. 2009)
CatK	↑↓	Baboon, Rat, Mouse	(Altiok, Yasumatsu et al. 2006; Knaapi, Lukkarinen et al. 2006; Knaapi, Kiviranta et al. 2014)
CatS	↑	Mouse	(Hirakawa, Pierce et al. 2007)
CatB	↑	Baboon	(Altiok, Yasumatsu et al. 2006)
CatH	↑	Baboon	(Altiok, Yasumatsu et al. 2006)
CatL	↑	Baboon	(Altiok, Yasumatsu et al. 2006)
TIMP1	↑	Mouse	(Piedboeuf, Johnston et al. 1994; Zhang, Zhu et al. 2008)
TIMP2	↑	Mouse	(Zhang, Zhu et al. 2008)

2.5.1 Cathepsin K

Cathepsins are papain family cysteine proteases with collagenolytic and elastinolytic properties (Garnero, Borel et al. 1998), and are known to be involved in several specific biological effects like apoptosis (Li, Rayford et al. 2004; Stoka, Turk et al. 2007), bone resorption (Kiviranta, Morko et al. 2005) and inflammation (Lang, Horler et al. 2000; Honey and Rudensky 2003). Cathepsin K (CatK) is one of the most potent collagenases and elastases, which is capable of matrix remodeling by degrading type I, II and IV collagen and elastin (Chapman, Riese et al. 1997; Buhling, Waldburg et al. 2000). In fibrotic adult mice lungs, increased CatK expression is mainly located to fibroblasts (Buhling, Rocken et al. 2004), while in newborn baboons suffering from BPD pulmonary CatK is mainly expressed in the perivascular areas (Altiok, Yasumatsu et al. 2006). Lack of CatK aggravates collagen deposition in bleomycin-induced lung injury in adult mice (Buhling, Rocken et al. 2004). Interestingly, CatK overexpression protected adult mice lungs from the peak fibrotic response at postnatal day (PN) 28 but not before (Srivastava, Steinwede et al. 2008). CatK has been suggested to control lung fibrosis while cytokine transforming growth factor beta TGF-beta may have regulative role to limit CatK overexpression (van den Brule, Misson et al. 2005) and on the other hand may induce the expression of vascular endothelial growth factor VEGF (Jeon, Chae et al. 2007).

In fact, it has been proposed that CatK inducing drugs like curcumin may be beneficial in the treatment of lung fibrosis (Zhang, Huang et al. 2011). CatK was originally identified in osteoclasts, but has been since found in various tissues, including the lungs (Rantakokko, Aro et al. 1996; Buhling, Waldburg et al. 2000; Buhling, Waldburg et al. 2002). Experimental evidence indicate that CatK is expressed in the bronchial and type II alveolar epithelial cells in adult and fetal lungs (Buhling, Waldburg et al. 2000; Buhling, Waldburg et al. 2002) and may be important for the physiological pulmonary matrix turnover and perinatal lung development (Chapman, Riese et al. 1997; Haeckel, Krueger et al. 1999; Buhling, Waldburg et al. 2000; Buhling, Waldburg et al. 2002). In addition, CatK appears to be upregulated at sites of inflammation and may contribute to extracellular matrix remodeling in inflammatory and

fibroproliferative disorders of the adult and newborn lungs (Chapman, Riese et al. 1997; Buhling, Rocken et al. 2004; Bose, Dammann et al. 2008). A recent investigation of atherosclerotic plaque formation showed that particularly CatK deficiency resulted in perivascular foam-cell formation, possibly through increased scavenger receptor B (CD36) activity (Lutgens, Lutgens et al. 2006). Interestingly, CatK has been suggested to be a marker of macrophage differentiation (Buhling, Reisenauer et al. 2001). CatK may have a modulatory role in the time-dependent regulation of cellular apoptosis during postnatal lung remodeling (Kresch, Christian et al. 1998).

In newborn baboons suffering from BPD-like disorder, CatK expression is up-regulated in the lungs, but mainly in the perivascular areas of the lungs indicating a role for CatK in controlling lung vascular balance (Altiok, Yasumatsu et al. 2006). Recent results also suggest that CatK deficiency impedes normal lung development by increasing fibroproliferation and alveolar wall thickness (Zhang, Leung et al. 2011). Since the role of CatK seems to be injury and age dependent, the exact role of CatK in the injurious process of the developing lungs is therefore still unclear.

3 AIMS OF THE PRESENT STUDY

To find out if

1. Cathepsin K expression is altered in developing BPD?
2. Cathepsin K gene deletion or its transgenic overexpression alter lung development in newborn mice?
3. Cathepsin K plays a role in oxygen-induced chronic lung injury in newborn mice?

4 MATERIALS AND METHODS

4.1 Pulmonary cathepsin K protein content of the newborn infants (I)

Cathepsin K protein levels were measured in repeated tracheal aspirate samples (TAF) (n=46) during the first two weeks of life in 13 intubated and ventilated preterm infants, treated for respiratory failure in the neonatal intensive care unit, University Central Hospital, Helsinki, between 1994 – 2002. Infants with major anomalies or with septic infections were excluded. BPD was defined as a need of supplemental oxygen at 36 weeks of postmenstrual age and chest radiographic findings typical for BPD. All infants were treated with antenatal glucocorticoids, but none of them received steroids postnatally during the study period. Parental informed consent was obtained, and the study was approved by the local Ethics Committee. Expression of cathepsin K protein (28 kDa) was measured by western blot analysis using an anti-human cathepsin K monoclonal antibody (Chemicon). Data were corrected with β-actin and for dilution by adjustment with the secretory component of immunoglobulin A (Cederqvist, Sorsa et al. 2001).

4.2 Animals and study design (I, II, III)

CatK-deficient (KO) and overexpressing transgenic (TG) mice were generated as earlier described (Kiviranta, Morko et al. 2005). CatK-deficient ($CatK^{KO/KO}$) C57Bl/6J background pups were produced by crossbreeding heterozygous $CatK^{WT/KO}$ mice. CatK transgenic ($CatK^{TG/TG}$) FVB/N background pups were produced by crossbreeding heterozygous transgenic $CatK^{TG/WT}$ mice. After the study period, genotyping for all mice was done as earlier described (Rantakokko, Aro et al. 1996). Heterozygous mice were excluded from further analysis. CatK WT, TG, and CatK KO newborn mice as well as newborn rats were housed in room air or in FiO2 90% for 7 or 14 days. All animals had free access to food and water. Dams were rotated between room air and oxygen chamber once a day to minimize the oxygen exposure of adult animals. Temperature, humidity, O_2-level and CO_2-level in the chamber were controlled twice a

day. Genotyping of all mice was done by PCR using primers specific for transgene constructs as previously described (Rantakokko, Aro et al. 1996). The study was approved by the local Ethics Committee in the University of Turku.

Newborn mice (II, III) and rats (I) were sacrificed on postnatal day (PN) 7 or 14 with an i.p. injection of pentobarbital (70 mg/kg, Mebunat^{R} 60 mg/ml, Orion, Finland) and tails of the mice were used for PCR genotyping (Kiviranta, Morko et al. 2005). The animals were weighed, chest wall was opened and lungs were removed, weighed and frozen with liquid nitrogen and stored in -80 C° for biochemical analyses. For histological analyses, trachea was cannulated with a 19G needle and the lungs were inflation-fixed with paraformaldehyde (pressure 20 cmH_2O), then removed and stored for 24 hours before embedding in paraffin.

4.3 RNA extraction and Northern hybridization (II, III)

Total RNA was extracted from frozen lung samples of newborn WT and CatK KO mice and studied as earlier described (Rantakokko, Aro et al. 1996). The signals were detected with Fuji Bas 5000 phosphoimager (Fuji, Tokyo, Japan).

4.4 Histology and morphometrical analysis (II,III)

For histological evaluation, the lungs were fixed in 4 % paraformaldehyde for 24 hours and then embedded in equal position in paraffin blocks for routine sectioning. Axially cut central sections were stained with hematoxylin – eosin (HE) for morphometrical analysis. Lung sections stained with HE were photographed with Zeiss Axiovert 200M microscope with an X10 objective lens. Ten image fields from one lung section per animal were used for the histological and morphometrical methods. Alveolar growth arrest is one of the most common findings in chronic lung injury (Coalson 2006). To investigate alveolar growth arrest mean alveolar size, thickness of saccular/alveolar septa, lung tissue fraction and secondary crest formation were analyzed. Quantification of distal airspace size was performed using the mean chord length as a measure of alveolar size (Bry, Whitsett et al. 2007). The digital images were analysed using Scion

Image software (Scion Corp., Frederick, MD) with a chord length macro (available from the US National Institutes of Health at http:\rsb.info.nih.gov/nih-image). A grid of parallel lines spaced at 20 μm was added to the images, and the intersections of lung tissue and grid lines were automatically indentified. The length of lines overlying alveolar space was then averaged as the mean chord length. To reduce optical and histological processing noise, chords less than 6 μm and greater than 250 μm were excluded. Areas of bronchiolar airways and blood vessels were excluded from the analysis. The same images were used to assess the thickness of saccular/alveolar septa (Bry, Whitsett et al. 2007). Straight lines (~ 30–40 per field, 300-500 per lung section) were drawn at 90° angles across the narrowest segments of septa of distal airspaces. The mean length of lines crossing the septa was determined using NIH Image software. Lung tissue fraction (tissue area / overall area) was evaluated with MCID M4 Image Analyzer software (Imaging Research inc, Ontario, Canada). Secondary crests were counted from the same images by identifying their characteristic structure as small ridges extending from both sides of the alveolar wall (II). Measurements were carried out blinded to group allocation.

4.5 Immunohistochemistry (II)

Histological lung sections were subjected to Leder-staining to visualize leukocytes in the lungs (II). To further analyze CatK expression and macrophage infiltration in the lungs, the lung sections were immunohistochemically stained with polyclonal goat anti-mouse CatK antibody (clone C-16, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA) (II, III) and monoclonal rat anti-mouse Mac-3 antibody, clone M3/84 (1:50, BD Pharmingen), respectively, and detected with biotinylated secondary antibody, avidin–biotin peroxidase (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) and Vector NovaRED (Vector Laboratories) substrate according to manufacturer's instructions. The number of Mac3-positive cells was counted from at least 10 fields of X40 lens in each lung section and the number of positive cells per mm^2 was calculated (II).

Separate lung sections were consecutively stained with macrophage specific Mac3 antibody and Oil Red O (Sigma, St. Louis, MO) to detect the lipid droplets in the pulmonary macrophages. Formalin fixed and paraffin embedded 5μm thick lung sections were deparaffinized and rehydrated. After routine staining with Mac3 antibody, slides were immersed in 100% propylene glycol for 10 minutes. Oil Red O was prepared by slowly dissolving powder in propylene glycol (0.7 g/100 mL), while heating the solution to 97°C. The solution was then filtered (Whatman No 2) and cooled, and then filtered again. The slides were incubated in Oil Red O for overnight and rinsed in 85% propylene glycol for 3 minutes after staining. Sections were counterstained with Mayers hematoxylin for 20 sec and gently rinsed in distilled water and mounted in aqueous medium.

4.6 Detection of collagen accumulation (I, II, III)

Picro-sirius red staining was used to evaluate the accumulation of collagen in the lungs. Paraffin embedded lungs were cut in 5 μm thick sections. Deparaffinized lung sections were incubated for 90 minutes in the 1 % picric acid solution, washed two times with acidified water, dehydrated and mounted in resinous medium. The lung sections were imaged using light microscope with an X20 objective lens.

4.7 In situ Detection of apoptotic cells (II, III)

In situ DNA nick-end labeling (TUNEL) for the detection of apoptotic cells was performed in paraffin wax sections, as described earlier (Lukkarinen, Laine et al. 2005). Apoptotic cells were counted in lung sections stained with the antidigoxigenin antibody. While TUNEL may have detected DNA fragments and random breaks of necrotic cells, the method is widely used for quantitative analysis and cell localization of apoptotic cells (Hotchkiss, Dunne et al. 2001). The results are expressed as the number of positive cells per mm^2 of tissue section area in at least ten fields of view of an X20 objective lens.

4.8 Measurements of oxidative stress (II)

4.8.1 Myeloperoxidase (MPO) activity

As a measure of pulmonary neutrophil influx and activation, tissue specimens of the lungs were initially frozen and measured for myeloperoxidase (MPO) activity. Before the biochemical analyses, tissue specimen were homogenized in 0.1 M Tris buffer (100 mg wet weight / mL) with 1 nM EDTA. MPO activity was assayed spectrophotometrically using a method in which the enzyme catalyzes the oxidation of 3,3', 5,5'-tetramethylbenzidine by H_2O_2 to yield a blue chromogen with a maximum wavelength of 655 nm (Suzuki, Ota et al. 1983).

4.8.2 Reduced Glutathione (GSH)

Lung tissue reduced glutathione (GSH) content, reflecting tissue antioxidative activity, was estimated by a colorimetric method, as described earlier (Saville 1996). Before the biochemical analyses, tissue specimen were homogenized in 0.1 M Tris buffer (100 mg wet weight / mL) with 1 nM EDTA and 10 µM indomethacin, pH 7.4. The results are expressed as nmol/mg protein.

4.8.3 Oxidized DNA (oxDNA)

Lung tissue DNA was isolated and purified by a DNA purification kit (NucleoSpin Tissue, Macherey-Nagel, Düren, Germany). Before the biochemical analyses, tissue specimen were homogenized in 0.1 M Tris buffer (100 mg wet weight / mL) with 1 nM EDTA and 10 µM indomethacin, pH 7.4. The pure DNA was hydrolysed to nucleotides with nuclease P1 and further to nucleosides with alkaline phosphatase. The nucleosides were separated by C18 reversephase column (Phenomenex Luna C18(2), 3 µm, 4.6 x 150 mm). The elution solution was 50 mM citric acid-sodium citrate buffer, pH 3.75, with 10 % methanol. The amount of 8-hydroxy-2´-deoxyguanosine (8-OH-dG) was determined using high pressure liquid chromatography (HPLC) equipped with an electrochemical detector (Shimazu, Japan) and deoxyguanosine (dG) was determined with UV-detector

(Shimatzu, Japan). The amount of 8-OH-dG, as an indicator of oxidatively modified DNA, was expressed as the number of 8-OH-dG per 10^5 dG.

4.8.4 Lipid peroxides (TBA-reactive material)

The thiobarbituric acid-reactive material (TBARM), a biomarker of oxidative stress, was analyzed to estimate tissue levels of lipid peroxides. Before the biochemical analyses, tissue specimen were homogenized in 0.1 M Tris buffer (100 mg wet weight / mL) with 1 nM EDTA and 10 μM indomethacin, pH 7.4. For the analysis of TBARM, tissue specimen were homogenized with phosphate buffer and heated together with a TBA solution (375 mg/ml) (Sigma) in a boiling water bath for 15 min. The tubes were then cooled and the absorbances measured at 535 nm (Sigma) (Ahotupa, Mantyla et al. 1997).

4.9 Gene expression of the lungs in microarray analysis (IV)

RNA amplifications were made from 500 ng total RNA with Ambion's Illumina RNA TotalPrep Amplification kit (cat no AMIL1791, Ambion Inc., Austin, TX, U.S.A.). In vitro transcription (IVT) was allowed to continue overnight (14h) and during the reaction cRNA was biotinylated. Both before and after the amplifications the RNA/cRNA concentrations where checked with Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, U.S.A.) and RNA/cRNA quality was controlled by BioRad's Experion electrophoresis station (Bio-Rad Experion Automated Electrophoresis System, cat.no: 700-7002, Bio-Rad Laboratories, Hercules, CA, U.S.A.)

1500 ng of each sample was hybridized to Illumina's Sentrix® Mouse-6 Expression BeadChips, at 58 °C overnight (19 h) according to Illumina® Gene Expression System -protocol, revision F (Illumina Inc., San Diego, CA, U.S.A.). Hybridization was detected with 1μg/ml Cyanine3-streptavidine, GE Healthcare Biosciences (cat no PA43001, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, U.S.A.). Chips were scanned with Illumina BeadArray Reader (factor 3.96) and the numerical results were

extracted with Illumina Bead Studio software 1.5.0.34 without any normalization or background subtraction.

4.10 Statistical analyses

Statistical analyses were performed with one- or two-way analysis of variance (ANOVA) and the Tukey´s post-hoc test, where appropriate. Results are expressed as means (SD). P values <0.05 were considered statistically significant (I). Time-dependent changes in CatK mRNA expression, morphometric analyses and measurement of oxidative stress were analyzed using two-way analysis of variance (ANOVA) and the Bonferroni´s post-hoc test (II). Survival data were compared using Logrank (Mantel-Cox) test (II). A p-value <0.05 was considered significant. With TG mice all statistical analyses were performed with two-way analysis of variance student t-test (ANOVA) and the Bonferroni´s post-hoc test except histological analyses were performed with student t-test (III). The results are expressed as means (SEM) (II, III). All statistical analyses were performed with Prism 4.0 Graphpad software (San Diego, CA) (I, II, III). The signal intensity values of gene expression were raw-wise normalized before analyzing the group differences with multiclass comparison and unpaired t-test of Significance Analysis of Microarray (Significance analysis for microarrays (SAM), Stanford University). After an analysis with 100 permutations, threshold Δ was assessed to receive the False Discovery Rate (FDR) of <5%, which was considered to be a biologically significant cutoff level. Contrast values of all significant genes were used for further analysis with complete hierarchial clustering (Cluster 3.0, Stanford University) to demonstrate the possible similarities in gene expression profiles between the groups or genes. Contrast value is standardized mean difference between the gene's expressions in that class, versus its overall mean expression. The clustering results were visualized with Java Treeview 1.1.0 software (Stanford University) and the genes from the clusters were linked to gene ontology using the GenBank (IV). To associate the gene expression profiles to the histological changes seen in earlier study, significantly altered genes where grouped by gene ontology using the GenBank.

5 RESULTS

5.1 Cathepsin K in newborn infants

The expression of cathepsin K in ventilated infants without BPD did not change during the study period. By contrast, patients developing BPD had initially (1-4d) similar values of CatK expression than those surviving without BPD, but thereafter the expression of CatK decreased significantly ($p<0.05$) with time and was down-regulated at 9-13 days, when compared to controls. Infants developing BPD had lower birth weight, lower initial Apgar scores and longer ventilation times compared to infants surviving without BPD.

5.2 Cathepsin K expression, histology and apoptosis in the lungs of air bred neonatal mice

Expression of pulmonary CatK mRNA in air-bred WT mice remained similar in FVB/N mice but decreased in C57Bl/6J from PN 7 to PN 14. There was 2-fold higher pulmonary CatK mRNA expression in air-bred TG mice which increased 4-fold at PN 14 when compared to WT mice at PN 14. As expected, no CatK mRNA expression was detected in the lungs of air-exposed CatK KO mice. Immunohistochemical staining of lung tissue revealed that the expression of CatK was mainly located in the alveolar macrophages, bronchial epithelial cells and alveolar walls of the newborn mice (Figure 3).

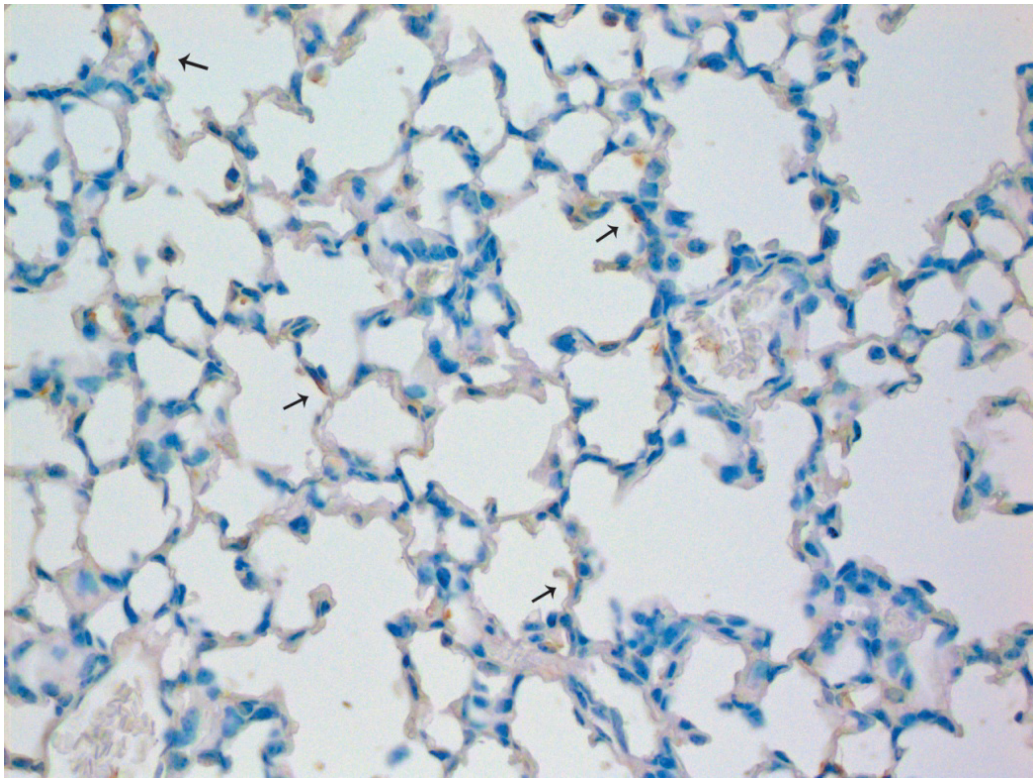

Figure 3: Cathepsin K staining of the lungs of an air-bred cathepsin K wild-type mouse at postnatal day 14. Arrows show the positive staining in the alveolar wall. Brown color indicates polyclonal goat anti-mouse cathepsin K antibody.

The lungs of air-bred WT mice demonstrated ongoing secondary crest formation, thinning of alveolar walls, and formation of alveoli at PN 7. Lung development of air-bred CatK KO mice was similarly ongoing, but the walls of distal airspaces were thinner at PN7 when compared to the WT mice. These changes were transient and alveolar chord length, lung tissue-air fraction and septal wall thickness were similar in these two groups of mice at PN 14 (Table 2). Overexpression of CatK in air-bred newborn mice resulted in large emphysematic distal airspaces at PN 7 and PN 14 indicated by increased alveolar chord length and decreased tissue-air fraction at PN 14, but the septal wall thickness and tissue fraction where similar to the WT mice (Table 2).

Table 2: Lung morphometrical analyses. Mean (SEM); *p<0.05 vs. air-bred cathepsin K wild-type (WT) mice. ** p<0.05 vs. oxygen exposed cathepsin K mice. *** p<0.05 vs. air-bred cathepsin K knock-out (KO) mice. † p<0.05 vs. air-bred cathepsin K transgenic overexpression (TG) mice.

			Air		O_2	
			WT	KO	WT	KO
			n=6	n=6	n=6	n=6
C57B1/6J	PN 7	Chord length (µm)	38.4 (1.01)	38.8 (1.10)	42.8 (0.51) *	45.8 (0.76) ***
		Lung tissue fraction (%)	39.0 (3.72)	32.4 (0.89)	26.3 (0.84) *	27.8 (1.00) ***
		Alveolar wall thickness (µm)	3.24 (0.14)	2.82 (0.08) *	2.45 (0.16) *	2.58 (0.05)
			n=6	n=6	n=6	n=6
	PN 14	Chord length (µm)	26.1 (0.61)	26.5 (0.61)		
		Lung tissue fraction (%)	33.77 (1.40)	33.80 (1.09)		
		Alveolar wall thickness (µm)	1.83 (0.07)	1.85 (0.09)		
			WT	TG	WT	TG
			n=4	n=6	n=4	n=6
FVB/N	PN 7	Chord length (µm)	54.4 (4.17)	66.0 (2.75) *	73.5 (7.97) *	69.6 (3.89)
		Lung tissue fraction (%)	45.3 (3.42)	39.2 (2.35)	34.3 (3.91) *	33.2 (2.58)
		Alveolar wall thickness (µm)	2.68 (0.08)	2.85 (0.11)	3.03 (0.20)	3.035 (0.02)
		Alveolar crest count (number of secondary crests per field)	125,1 (7,2)	115,9 (9,0)	46,5 (2,0) *	60,8 (3,3) † **
			n=5	n=6	n=2 N/A	n=6
	PN 14	Chord length (µm)	45.7 (1.91)	55.3 (2.06) *	97.8 (1.55)	85.9 (7.21) †
		Lung tissue fraction (%)	42.9 (1.26)	35.1 (2.33) *	26.2 (0.75)	29.1 (3.16)
		Alveolar wall thickness (µm)	2.33 (0.06)	2.21 (0.09)	3.03 (0.32)	2.65 (0.18) †
		Alveolar crest count (number of secondary crests per field)	112,0 (8,0)	123,8 (4,7)	33,2 (2,9)	31,7 (1,3) †

Some TUNEL-positive cells were seen in the lungs of air-bred WT mice at PN 7 and PN 14. The number of TUNEL-positive cells in the lungs of air-bred CatK TG mice was increased massively at PN 14 while lungs of the CatK WT mice were similarly stained compared to PN 7. Interestingly, while occasional TUNEL-positive cells were seen in the lungs of air-bred WT mice at PN 7 and PN 14, the number of TUNEL-positive cells in the lungs of air-bred CatK KO mice was lower at PN 7 and higher at PN 14 than the corresponding numbers of WT littermates. CatK overexpression or deficiency did not have an effect on body weight gain, relative heart weight or relative lung weight in air-bred neonatal mice on PN14 compared to WT mice.

5.3 Cathepsin K expression, histology and apoptosis in oxygen-induced lung injury

In agreement with the pulmonary finding in newborn infants with BPD, hyperoxic lung injury in newborn rats resulted in low expression of CatK mRNA at PN14, but not before. This same suppression in CatK mRNA expression was not seen in WT mice. CatK mRNA expression was increased 2-fold in the lungs of oxygen-exposed TG pups at PN 7 and 4-fold at PN 14 when compared to the oxygen-exposed WT controls. Although the expression level of CatK mRNA remained unchanged in oxygen exposed WT mice, a stronger staining of pulmonary CatK protein was detected in the bronchial epithelium, alveolar type II epithelial cells and macrophages stronger when compared to the air bred controls by immunohistochemistry.

Exposure of WT newborn mice to hyperoxia increased alveolar chord length, and decreased alveolar wall thickness, lung tissue fraction and secondary crest count at PN 7 and PN 14, while at PN 14 both chord length and septal wall thickness were increased when compared to the air-bred controls (Table 2). In CatK KO mice, exposure to hyperoxia did not significantly decrease the alveolar wall thickness, but resulted in highly increased mean alveolar chord length indicating impaired alveolarization (Table 2). In contrast to the WT and CatK KO mice, in CatK TG mice oxygen-exposure for seven days did not increase alveolar chord length and disrupt alveolarization. The secondary crest formation was decreased in hyperoxia-exposed TG mice when compared to air bred mice, but significantly less than in oxygen exposed WT mice (Table 2). Analyses in WT mice at PN 14 were impossible to carry out because only 2 mice were obtained for analyses but alveolar enlargement was obvious in these mice. Although overexpression of CatK seemed to prevent oxidative damage in newborn lung at PN7, the chord length was similarly increased at PN14 when compared to controls (Table 2).

Pulmonary apoptosis was similar in hyperoxia-exposed TG and WT mice at PN 7 and 14. Hyperoxic exposure for one week increased the number of TUNEL-positive cells in CatK KO mice from the room air level but there was no difference in the number of

TUNEL-positive cells between the CatK KO and WT mice after one week of hyperoxic exposure.

Hyperoxic challenge decreased total body weight, relative lung weight and relative heart weight to a similar degree in both CatK KO and WT newborn C57Bl/6J mice at PN 7 while in FVB/N TG and WT mice total body weight relative lung weight and relative heart weight was not altered by oxygen challenge. Relative lung weight was transiently down-regulated at PN 7 in hyperoxia-exposed CatK TG and WT mice but at PN 14 relative lung weights were similar.

Excessive hyperoxia caused respiratory distress, characterized by increased respiratory rate and difficulties in breathing, in both WT and CatK KO mice after 6 days of oxygen exposure. While 20% of hyperoxic WT mice survived for 2 weeks, respiratory distress in CatK KO newborn mice was rapidly progressing and fatal between 7 and 9 days of life and none of the CatK KO mice survived for two weeks. In a different mouse strain, survival of CatK TG mice was 83 % while 63 % of WT mice survived until PN14 (Fig. 4).

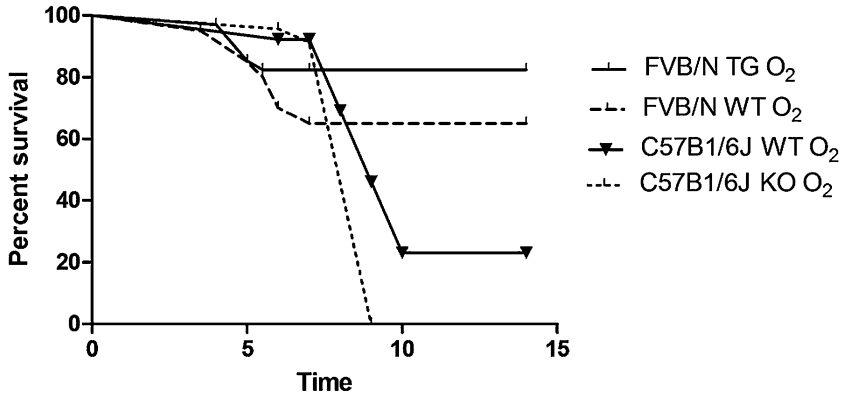

Figure 4: Survival curves of hyperoxia-exposed cathepsin K transgenic overexpressing (TG) and knock-out mice (KO).

5.4 Pulmonary fibrosis in neonatal mice and rats

Picro-sirius red staining revealed that collagen deposition was localized in the alveolar tips of the lungs similarly in air-bred CatK WT, TG and KO neonatal mice at PN 7.

Oxygen exposure for one week caused an apparently slight accumulation of collagen in the alveolar walls in WT mice, but interestingly, CatK deficiency did not aggravate collagen deposition in the lungs of hyperoxia-exposed mice. Oxygen exposure for two weeks caused accumulation of collagen in the alveolar walls in WT and TG mice, but CatK TG mice had no significant variation in collagen deposition in the lungs of hyperoxia-exposed mice. However, in oxygen-exposed rats, down-regulation of pulmonary CatK was accompanied by increased amount of extracellular collagen deposition, analyzed by tissue hydroxyproline content, at 14 days.

5.5 Accumulation of inflammatory cells in lungs of neonatal mice

Leder-staining revealed no significant pulmonary influx of neutrophils in hyperoxia-exposed CatK KO or WT neonatal mice at PN 7. The levels of MPO remained unchanged after oxygen challenge for 7 days in both study groups compared to air-bred controls. On the other hand, hyperoxia increased the number of pulmonary macrophages (Mac-3-staining) in the lungs of WT mice at PN 7 and intensified pulmonary macrophage accumulation in CatK KO mice. Double-staining with Mac-3 and Oil-Red-O additionally demonstrated few macrophage-derived multinucleated giant cells with lipid filled cytoplasm in the lungs of hyperoxia-exposed wild-type mice whereas their number was higher in CatK KO neonatal mice lungs (IV).

5.6 Lung oxidative stress in neonatal mice

Measurement of oxidative stress was mainly carried out with protein content analyses. Protein leak is common in affected lungs. However, we did not see any protein leakage in histological analysis and, thus, we assumed that there was no significant difference in protein contents between the study groups. As suggested, we reviewed the protein contents of all studied samples and found no difference in the protein contents between the genotypes. Furthermore, the lung-to-body weight was decreased in hyperoxia-exposed mice supporting our findings that the lung injury is mainly due to growth failure and not due to excessive lung parenchymal injury and vascular leakage.

Hyperoxia did not alter lung tissue content of reduced GSH in WT mice, but was associated with increased pulmonary GSH levels in the CatK KO mice at PN 7, potentially indicating increased oxidative stress in these lungs. The protein contents of the lung homogenates were similar in all mice suggesting minimal vascular leakage due to hyperoxic exposure. Oxidation of lipids, analyzed by TBARM, was enhanced in the lungs of oxygen-exposed WT and CatK KO mice at PN7, but there was no difference between the genotypes (II). Lung tissue DNA oxidation and MPO levels remained unchanged in all study groups.

5.7 Microarray analysis

Microarray analysis was made from lung samples of 3 mice in each group. Group size is minimal but the expression profiles were similar within the groups. Furthermore, a hierarchical clustering analysis grouped all animals in the right treatment groups according to the expression profile. Since microarray analysis was done from whole lung samples, local and cell specific differences in expression profile may be possible.
A multiclass analysis between air-bred or hyperoxia-exposed WT and CatK KO mice (SAM, False Discovery Rate, FDR<5%, $p<0.05$) demonstrated that overall 134 genes were significantly altered. With stricter inclusion criteria (FDR 0, $p<0.001$) we found 20 up-regulated and 9 down-regulated genes between all study groups. Of these genes, the largest differences were found in apoptosis-related genes Ccng1, Edar2, Cdkn1a, plau, inflammatory response plau, Loxl4, Vtn, and DNA damage response Ddit4l, Cdkn1a.

Then we evaluated more specific group differences pair wise with SAM unpaired t-test. The analysis demonstrated that the pulmonary expression of 107 genes were significantly altered between air-bred and oxygen-exposed WT mice (False Discovery Rate, FDR<5%, $p<0.05$). With stricter inclusion criteria (FDR 0, $p<0.001$) only 11 significantly up-regulated and 13 significantly down-regulated genes were found. The most down-regulated gene was Chloride channel calcium activated 3 (Clca3, fold change 0.04, $p=0.02$) and the most up-regulated gene was Cyclin-dependent kinase inhibitor 1A (Cdkn1a, fold change 10.5, $p=0.02$). The most significant changes were

seen in genes related to apoptosis, inflammatory response, oxidative stress, DNA damage response and proteolysis.

Finally, we compared the gene expression differences between the oxygen-exposed WT and CatK KO mice. Only one significantly altered gene was found, MSR 2 (Fold difference 0.55, FDR 0, $p<0.001$). While MSR2 expression was increased due to hyperoxic exposure in WT mice, in CatK KO mice MSR2 levels were down-regulated.

6 DISCUSSION

6.1 Cathepsin K expression in bronchopulmonary dysplasia

In previous studies, proteases, like matrix metalloproteinases, are mainly increased and antiproteases are decreased in TAF samples of newborn infants developing BPD (Table 1). Since CatK has been found in epithelial cells of the developing bronchi of newborn infants and its expression is upregulated parallel with alveolar and bronchial lumen formation, the role of CatK in the developing lungs may be multifaceted (Buhling, Waldburg et al. 2000; Buhling, Waldburg et al. 2002). CatK is suggested to have an influence on matrix remodeling of the lungs under physiological and pathological conditions (Buhling, Waldburg et al. 2000). In our study, CatK protein levels were lower in TAF samples of newborn human infants developing BPD (IV). Thus, we hypothesized that CatK as a collagenase and elastase may have an important role in developing lungs under hyperoxic stress. Infants in our study had ventilatory treatment so the impact of airway pressure to the CatK expression cannot be excluded. In previous studies, proteases are mainly interpreted to be harmful and suggested to aggravate the injury progression in infants developing BPD (Cederqvist, Sorsa et al. 2001). However, possible connection between development of the BPD in newborn lungs and protease-antiprotease balance and mechanisms behind this phenomenon needs further investigations.

In this study, we used strict selection criteria with infants developing BPD. Due to the strict selection criteria, both study groups had a limited number of infants that may have influenced our results. Although the treatment strategies of BPD have improved, the incidence of BPD has remained the same or even increased (Jensen and Schmidt 2014). More premature infants survive and consequently the poorly developed lungs are the main cause for BPD (Bose, Van Marter et al. 2009). Experimental models that mimic the present clinical picture of the BPD, such as preterm baboons delivered after 67% of gestation or preterm lamb models, have widened our knowledge of the pathogenetic mechanisms of new BPD (Walther, Jobe et al. 1998; Coalson, Winter et al. 1999). Nevertheless, epithelium of the respiratory tract is exposed to 21% oxygen

after birth which may even be harmful to poorly developed lungs and induce the alveolar growth arrest (Buczynski, Maduekwe et al. 2013). While even normal oxygen exposition may predispose preterm newborn lungs to injury, hyperoxic lung injury models still provide a good tool investigating the specific molecular mechanism associated with BPD.

6.2 The role of cathepsin K in newborn mice lung development

Since CatK is a potent collagenase and elastase, we hypothesized that pulmonary CatK may modify the development of newborn lungs. CatK is expressed in type II alveolar epithelial cells, bronchial epithelial cells and macrophages in newborn mouse lungs (II,III) (Buhling, Gerber et al. 1999). Recent studies suggest that CatK is involved in postnatal lung development by modifying the extracellular matrix protein compounds and the maintenance of the airway structural integrity (Zhang, Leung et al. 2011). In our study, CatK deficiency was associated with transient thinning of the alveolar walls (II), lower number of TUNEL positive cells at PN 7 and higher at PN 14 (II). Overexpression of CatK resulted in enlarged distal airspaces with decreased tissue/air ratio and increased number of TUNEL-positive cells (III). Potentially these findings may indicate modified postnatal development of the newborn lungs. Although the mechanism remains unclear, altered pulmonary development could be mediated by increased macrophage activation (Blackwell, Hipps et al. 2011), altered type II pneumocyte function or modification of the extracellular matrix by cathepsin K (Kinsella, Greenough et al. 2006). All together, these findings suggest a potent role for CatK in the development of newborn lungs and are in line with earlier findings (Zhang, Leung et al. 2011).

6.3 The role of cathepsin K in lung development during hyperoxic stress

Cathepsin K KO and TG mice have been used previously to study lung fibrosis (Buhling, Rocken et al. 2004; Srivastava, Steinwede et al. 2008). In the first study, we used rats in a model of hyperoxia-induced lung injury but transgenic and knock-out models were mice. We chose hyperoxia-induced lung injury as a model of BPD in newborn mice because it

is widely used, morphological alterations are well known (Mao, Gundavarapu et al. 2008) and the developmental stage of the newborn mouse lungs is in the saccular phase at the time of birth, that corresponds the newborn infant born on 24-28 weeks of gestational age (Amy, Bowes et al. 1977; Bhandari 2014). We obtained a limited number of study animals because of the study design, many study groups and Mendelian inheritance as described in the materials and methods section. We used two different strains of mice to study the effect of CatK overexpression and CatK deficiency in hyperoxic lung injury. TG mice were of FVB/N background while knock-out mice were of C57Bl/6J background. While the reactions to hyperoxic injury are eventually rather similar in different mouse strains, there are some differences in oxygen tolerance and the extent of hyperoxia-induced injury in different time points (Whitehead, Burch et al. 2006). Thus, comparison between the different mouse strains should be done cautiously.

The developing lungs are most vulnerable to hyperoxic stress in the saccular phase (Jobe 1999). Hyperoxic-exposure may result in fibrosis, enlarged airways and increased influx of inflammatory cells in the lungs of newborn mice (Pappas, Obara et al. 1983). These findings were obvious also in our studies of newborn mice and rats (I, II, III). In addition, we observed slight fibrosis that concentrated in the alveolar tips.

Pulmonary CatK mRNA expression was down-regulated in hyperoxia exposed newborn rats (I). This finding is in contrast to a study of preterm baboons with an experimental model of BPD that demonstrated increased CatK protein and mRNA levels in injured lungs (Altiok, Yasumatsu et al. 2006). Furthermore, in hyperoxia-exposed newborn mice, the CatK mRNA expression had a slight trend to increase (III) which underlines the multifaceted and time dependent role of CatK in lung development and injury. In line with this, as summarized in Table 1, previous studies have found an inconsistent role for proteases in different lung injury models. These variations may be due to different biological strain qualities or time dependent changes related to different developmental or injury states of the maturing lungs. In addition, measurement of mRNA levels may not reflect actual enzyme activity. However, tissue CatK protein levels of same CatK TG mice strain were studied in previous study demonstrating comparable protein levels when compared to the CatK mRNA

expression levels (Srivastava, Steinwede et al. 2008). This present work showed contradictory results while in the human newborn infants and in the laboratory rats enzyme activity and the mRNA levels were downregulated respectively, and in the hyperoxia exposed CatK gene manipulated mice CatK mRNA levels were upregulated.

Hyperoxia decreases survival rates in neonatal animals (Frank, Bucher et al. 1978). Newborn mice survival rates are demonstrated to be strain specific (Johnston, Stripp et al. 1998). Hyperoxia decreased survival in both strains and all genotypes in our study. However, CatK KO mice died within the first 9 postnatal days while 20% of C57Bl/6J WT mice survived at PN 14 (II). In FVB/N strain survival curves did not differ while survival proportions at PN14 of oxygen-exposed WT and TG pups were 65% and 82%, respectively (III). These results suggest a protective role for CatK in oxygen induced lung injury, even when strain specific differences are taken into consideration.

Accumulation of inflammatory cells in the lungs is a feature in hyperoxic lung injury model in rodents (Pappas, Obara et al. 1983). However, in our models, we found only increased macrophage influx, and we could not demonstrate significant neutrophil activity within the lungs of oxygen-exposed newborn mice (II). This discrepancy between present and earlier findings may be explained by different oxygen concentrations between different study models, follow up times or strain differences.

Oxidative stress in hyperoxia-exposed lung may cause significant damage to the immature lungs (Fellman and Raivio 1997). Chronic hyperoxic exposure increases the amount of reduced glutathione to protect the lungs against oxidative stress (Chessex, Lavoie et al. 1999). In line with these observations the content of reduced glutathione was increased in the present oxygen-exposed CatK KO mice lungs suggesting increased oxidative stress (II). Activated macrophages in the newborn lungs may be the source of oxidative stress and thereby disrupt alveolar development (Blackwell, Hipps et al. 2011). Lungs of the CatK KO mice contained high number of macrophages and multinucleated giant cells that together with increased reduced glutathione content associate macrophages as a potential source of oxidative stress also in the present injury model (II). CatK may control macrophage activity (Buhling, Reisenauer et al.

2001) or participate in more direct way to antioxidative functions, but these contemplations still need further investigations.

Earlier studies have suggested a central role for CatK in controlling the formation of pulmonary fibrosis in adult lungs. Hyperoxic lung injury models in rodents have shown that fibrosis is not a central feature seen in newborn mice or rats with this injury model during the first 2 weeks of life. Oxygen exposure in newborn rats is supposed to lead to significant fibrosis only after 21 days (Chen, Wang et al. 2007). In line with this finding, our both strains of WT mice did not result in excess fibroproliferation during the first two weeks of life in hyperoxic milieu (II, III). Thus, we could not see any significant preventive effect of CatK overexpression in the present injury model as was seen in bleomycine-induced lung injury in adult mice. In adult mice, CatK overexpression reduced bleomycin induced fibrosis in lungs at 4 weeks post-treatment but the effect was not seen earlier (Srivastava, Steinwede et al. 2008).

MSR 2 has been suggested to control lipid intake in macrophages with scavenger receptor B (CD36) (Tabas, Williams et al. 2007). MSR 2 macrophage specific overexpression resulted in increased foam cell formation (de Winther, van Dijk et al. 1999). Controversially, in our study MSR 2 mRNA expression was down-regulated and foam cell formation was increased in the lungs of CatK knock-out mice after 7 days of hyperoxic-exposure (IV) suggesting that not only MSR 2 but also probably multiple other proteins are involved in the regulation of lipid intake of macrophages in CatK KO mice. In line with a previous finding in mice, CatK deficiency results in excessive foam cell formation in atherosclerotic plaques (Lutgens, Lutgens et al. 2006). Furthermore, inspection of other macrophage scavenger receptors revealed that expression of lysyl oxidase-like 4 (Loxl4) was significantly increased in hyperoxia exposed lungs independently from the genotype proposing that only MSR II gene expression was altered by CatK deficiency (IV). We suggest that macrophage-derived multinucleated giant cells, induced by CatK deficiency, are detrimental to affected newborn lungs. However, more investigations are needed to explore the possible interplay between macrophages and CatK and its influence in lung development.

7 SUMMARY AND CONCLUSIONS

While we demonstrated lower CatK protein levels in newborn infants developing BPD and suggested a protective role for CatK in chronic lung injury, too much of CatK may cause pulmonary emphysema and thus the right balance of protease activity in the lungs may be of significance for lung development and injury repair.

In developing mouse lungs, CatK deficiency transiently thinned alveolar walls, indicating accelerated postnatal lung maturation (II). The change in lung development was associated with altered lung cell apoptosis. Overexpression of CatK resulted in enlarged distal airspaces with decreased tissue/air ratio and increased number of apoptotic pulmonary cells at PN 14 (III). These findings support the modulatory role for CatK in normal lung development and homeostasis.

Lungs of the CatK KO mice contained a high number of macrophages and multinucleated giant cells indicating intensified inflammatory response and probable oxidative stress (II). Overexpression of CatK preserved lung structure in hyperoxia-induced lung injury for 7 days, but not longer (III). Although expected, we did not find any change in the lung fibroproliferation in any of the phenotypes studied (II, III).

Further analysis of CatK KO and WT mice by microarray analysis showed that oxygen exposure changes the expression of numerous genes associated with inflammatory response, oxidative stress, DNA damage response, proteolysis and apoptosis. Interestingly, only one gene, macrophage scavenger receptor II (MSRII) was significantly altered between the oxygen-exposed CatK KO and WT lungs (IV). While the MSRII may be related to the foam cell formation seen in CatK KO lungs, the function of MSR2 in hyperoxia-induced lung injury needs more investigations.

These findings suggest that CatK has a modulatory role in normal lung development and insufficient production of CatK may aggravate oxygen-induced lung injury. Thus, the unique role of each protease has to be taken into account when developing novel modes of approaches against lung injury.

8 FUTURE DIRECTIONS

The role of proteases in newborn lung injury has been unclear, but earlier investigations have suggested mainly deleterious role for all proteases. Each protease may have a distinct role in lung development and thus it should be carefully evaluated which proteases are harmful and which are not. We demonstrated that CatK has a modulator role in lung development and its action in the injured newborn lungs may be more beneficial than harmful. Thus, instead of finding ways to inhibit different proteases to improve the outcome from BPD, the research should be also focused on balancing out effects of proteases involved in oxygen induced lung injury.

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